Chinese Herbs (*Lonicera japonica* and *Ganoderma lucidum*) Enhance Non-Specific Immune Response of Tilapia, *Oreochromis niloticus*, and Protection Against *Aeromonas hydrophila*

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ABSTRACT

Three diet variations, in addition to control (no herbs), were used to determine the effect of Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) on non-specific immune response of tilapia. These contained 1.0% of *Lonicera*, 1.0% of *Ganoderma* and a mixture of *Ganoderma* (0.5%) and *Lonicera* (0.5%). The diets were fed for 3 weeks. The respiratory burst activity of blood leukocytes, phagocytosis, plasma lysozyme, total protein and total immunoglobulin were monitored. Following three weeks after feeding, fish were infected with *Aeromonas hydrophila* and mortalities recorded. The results of this study showed that feeding tilapia with Ganoderma and Lonicera alone or in combination enhanced phagocytosis by blood phagocytic cells during the whole experimental period and stimulated lysozyme activity after two weeks. Respiratory burst activity of phagocytic blood cells, total protein and total immunoglobulin in plasma were not enhanced. Both herbs when used alone or in combination increased the survival of fish after challenge with *A. hydrophila.* The highest mortality was observed in control fish – 58% and fish fed with Lonicera extract – 43%, while 30% of fish died in the group fed with Ganoderma and the lowest mortality (21%) was observed when fish were fed with a combination of two herbs. Thus, it can be concluded that the herb extracts added to diets acted as immunostimulants and appeared to improve the immune status and disease resistance of fish.

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INTRODUCTION

Diseases caused by *Aeromonas hydrophila* are some of the most widespread in freshwater fish culture. Septicaemia caused by motile aeromonads is a ubiquitous problem that affects fishes found in warm, cool, and cold fresh water around the world (Plumb, 1999). The bacterium, *A. hydrophila* has been associated with disease in carp, eels, milkfish, channel catfish, tilapia and ayu and can also be an opportunist in stress-related diseases in salmonids (Miyazaki and Jo, 1985; Rahman *et al*., 1997; Plumb, 1999). Antibiotics are fequently used to control disease caused by this bacteria, but there is an increasing risk of developing antibiotic resistant strains of bacteria. Vaccines are being developed against *A. hydrophila* and atypical *A. salmonicida* but these are not yet commercially available and as *A. hydrophila* is such a heterogeneous species (multiple strains), vaccine development is extremely complex.

Our research is directed in an alternative, promising area. Herbs can act as immunostimulants, conferring early activation to the non-specific defense mechanisms of fish and elevating the specific immune response. Herbs have been used as medicine and an immune booster for humans for thousands of years in China. Recently, a growing interest has developed in using herbs in animal feeds by both researchers and feed companies. Chinese herbs contain many immunologically active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oils, which can enhance immune functions. Chinese herbs have been used as medicine to treat different fish diseases in China for many years. Herbs have also been used in the other countries for control of shrimp and fish disease, and successful results have been reported in Mexico, India, Thailand and Japan (Auro de Ocampo, 1993; Dey and Chandra, 1995; Direkbusarakom, 1996; Logambal and Michael, 2000). Recently, there has been increased interest in the immune stimulating function of some herbs in aquaculture. The non specific immune functions such as bacteriolytic activity and leukocyte function were improved by some mixtures of Chinese herbs in shrimp (*Penaeus chinensis*) and tilapia (Luo, 1997; Chansue *et al*., 2000).

Two herbs were selected for the current study: *Lonicera japonica* and *Ganoderma lucidium*. Tilapia (*Oreochromis niloticus*) were fed with the extracts of Chinese herbs either alone or in combination for three weeks to investigate the effect of these substances on the non-specific immune response of tilapia and to examine protection levels against a challenge by *A. hydrophila.*.

MATERIALS AND METHODS

Fish

Tilapia (*O. niloticus)* (52.5±3.50g) were acclimatised in a recirculation system at the Research Institute for Fisheries, Aquaculture and Irrigation (HAKI, Szarvas, Hungary). Fish were fed with a dry feed, produced in the experimental milling facility of the institute and kept in 2000 l fibreglass tanks with water temperature maintained between 22-23^{o}C.

Herbal extracts

Lonicera extract containing 25% of chlorogenic acid, and *Ganoderma* extract containing 30% of *Ganoderma* polysaccharide were commercial products obtained from Xuancheng Baicao Plants Industry and Trade Ltd. China.

Experimental design and sampling procedure

Experiments were performed in the recirculation system of HAKI, Szarvas, Hungary. Batches of three-month old tilapia with an average initial weight of 52.5±3.50 g were held in 100 l fibreglass tanks. Fish were divided onto 8 groups, each group contained 60 fish. Water temperature and pH were constant (22-23^{o}C; pH 8.5) during the experimental period, and dissolved oxygen was maintained at 80-90% of saturation. Water flow was maintained at 7 l/min.

Fish were fed *ad libitum* 6 times daily with the appropriate pelleted feed using an automatic feeder. Three diet variations, in addition to control (no herbs), were used. These contained 1.0% of Lonicera, 1.0% of *Ganoderma* and a mixture of *Ganoderma* (0.5%) and *Lonicera* (0.5%). Each group was fed in duplicate. The diets were fed for 3 weeks. Blood samples (6 fish/group) were collected from caudal vein one, two and three weeks after start of feeding. Heparin was used as an anticoagulant. Individual fish were sampled only once to avoid any influence on the assays due to multiple bleeding and handling stress on the fish.

Separation of leukocytes from the blood

Leucocytes for assay were separated from each blood sample by density-gradient centrifugation. One ml of histopaque 1.119 (Sigma) containing 100 µl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. One ml of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 ml of blood was carefully layered on the top. The sample preparations were centrifuged at 700 g for 15 min at 4°C. After centrifugation, plasma was collected and stored at -80°C for future analysis. Separated leukocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to $2x10^6$ viable cells/ml.

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by reduction of ferricytochrome c (Secombes, 1990). Briefly, 100 µl of leukocyte suspension and an equal volume of cytochrome C (2 mg/l in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma) at 1 µg/ml were placed in triplicate in microtiter plates. To test specificity another 100 µl of leukocyte suspension and solutions of cytochrome C containing PMA and superoxide dismutase (SOD, Sigma) at 300 U/ml were prepared in triplicate in microtiter plates. Samples were then mixed and incubated at room temperature

for 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nmoles O_2^- by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for each fish, and converting O.D. to nmoles O_2^- by multiplying by 15.87. Final results were expressed as nmoles O_2^- produced per 10^5 blood leukocytes.

Phagocytosis assay

Phagocytosis activity of blood leukocytes was determined spectrophotometrically by the method of Seely *et al.,* (1990). This assay involves the measurement of congo red-stained yeast cells that have been phagocytosed by cells. To perform the assay, 250 µl of the leukocyte solution was mixed with 500 µl of the congo red-stained and autoclaved yeast cell suspension (providing a yeast cell: leukocyte ratio of 40:1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml of ice-cold HBSS was added and one ml of percoll (1.055) was injected into the bottom of each sample tube. The samples were centrifuged at 850 g for 5 min to separate leukocytes from free yeast cells. Leukocytes were harvested and washed twice in HBSS. The cells were then resuspended in 1 ml trypsin-EDTA solution (5.0 g/l trypsin and 2.0 g/l EDTA, Sigma) and incubated at 37°C overnight. Absorbance of the samples was measured at 510 nm using trypsin-EDTA as a blank.

Lysozyme assay

Plasma lysozyme activity was measured spectrophotometrically by the method of Sankaran and Gurnani (1972). The lysozyme substrate was a 0.02 % (w/v) suspension of Micrococcus lysodeikticus in phosphate buffer (0.05 M, pH 6.2). Lyophilised hen egg white lysozyme was used as a standard. A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25°C. The results were expressed as mg/ml equivalent of hen egg white enzyme activity.

Total protein

Plasma total protein was measured by the biuret method using a commercially available kit (Reanal, Hungary)

Total immunoglobulin

Plasma total immunoglobulin was measured using the method described by Siwicki *et al*., (1994). Analysis of total immunoglobulin in plasma is based on the biuret method. However, primary separation of immunoglobulins from the plasma was achieved by precipitation with polyethylene glycol (PEG) and the resulting supernatant analysed. To perform the assay 100 µl of the plasma was combined with 100 µl 12% PEG and incubated at room temperature for 2 hr while being continuously mixed. Following incubation the mixture was centrifuge for 10 min at 400 g and total protein concentration in the supernatant was determined by the biuret method. Total immunoglobulin value for individuals was calculated from total protein value less the quantity of protein in the supernatant.

Bacterial challenge

Groups of 30 fish from each treatment were challenged by intraperitoneal injection after three weeks of feeding. *A. hydrophila* (strain B-02/12 Bacteriology Laboratory, Institute of Aquaculture University of Stirling, Scotland), isolated in Bangladesh was used as the challenge strain. The bacteria were cultured in tryptone soya broth (TSB) overnight at 28ºC, washed twice with sterile phosphate buffered saline (PBS) and then resuspended in PBS. The concentration of the suspension was determined spectrophotometrically using a pre-made standard curve relating concentration to absorbance at 610 nm. The concentration of bacterial suspension was adjusted accordingly with sterile PBS, prior to performing challenges.

A preliminary challenge using an unrelated, but similar, group of tilapia was performed to establish the LD_{50} dose of the bacterium prior to performing the challenge of fish under study. The LD_{50} was found to be 3×10^6 cells/ml and was used at this rate for the subsequent challenge. Fish were injected intraperitoneally (i.p.) with 0.1 ml of the bacterial suspension. Mortalities were observed for 10 days. Bacterial swabs were taken from the kidneys. All the surviving fish were killed using an overdose of anaesthetic.

Statistics

Results are presented as the average ($\pm$ standard error) for five fish, and were compared at each time point using one way ANOVA and Dunn's multiple range tests (Sigma Stat 3.2). Significant differences between experimental groups were expressed at a significance level of $P<0.05$.

RESULTS

The non-specific defence mechanism values for the fish fed with herb extracts are shown in Figures 1-5. Phagocytic activities of leukocytes were elevated for the whole period of the experiment and each treatment showed values higher than untreated control, the highest value was measured on week three in the group fed a combination of herbs (Figure 1). Herb supplementation in tilapia had no effect on respiratory burst activities of isolated phagocytic cells for the duration of the experiment. The production of oxidative radicals measured during 3 weeks of feeding the fish with all diets remained on the same level (Figure 2). The plasma lysozyme activities were significantly higher after two weeks in groups fed with herb extracts. However, there were no significant differences between treated groups (Figure 3). There were no significant changes in plasma protein (Figure 4) and total immunoglobulin during the whole experiment (Figure 5).

The results from the virulent pathogen challenges are shown in Figure 6. The highest mortality was observed in control fish (–58%) and fish fed with *Lonicera* extract (–43%), while 30% of fish died in the group fed with *Ganoderma* and the lowest mortality (21%) was observed when fish were fed with a combination of two herbs.

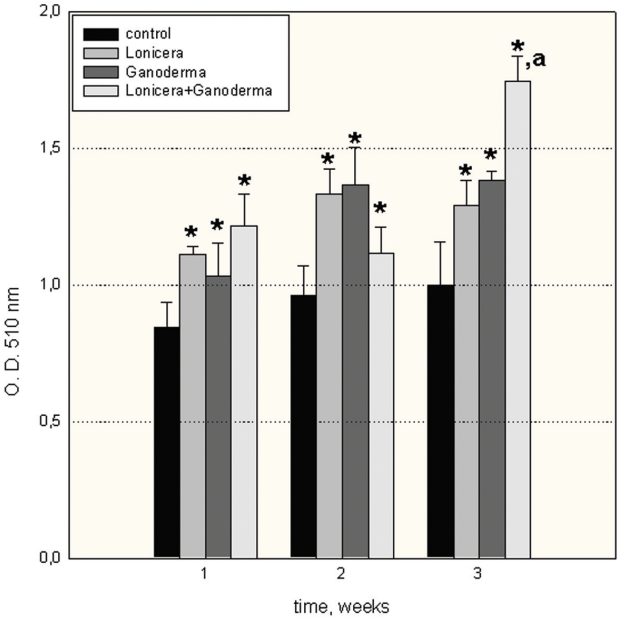

Figure 1. Phagocytic activity of isolated phagocytic cells in tilapia in control group and groups fed diets containing different kind of herbs. Data is expressed as the mean of six fish±SEM. Significance differences ($P<0.05$) from the untreated control are indicated by asterisks. Significant differences among group are indicated by letters.

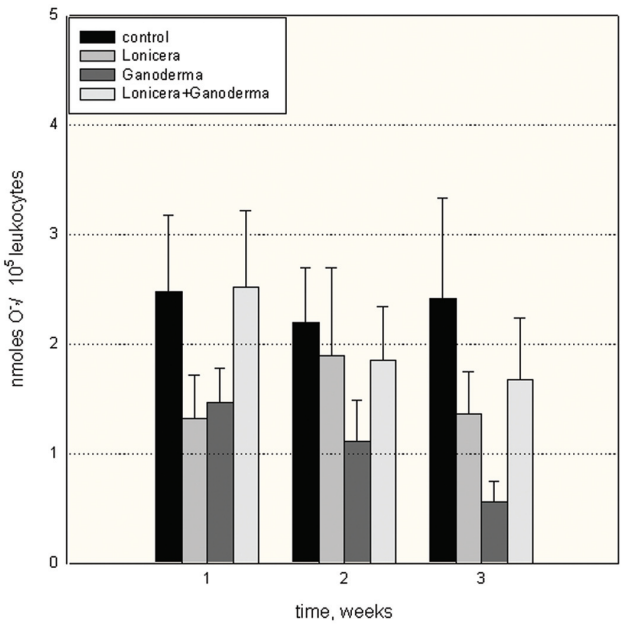

Figure 2. Changes in respiratory burst activities of phagocytic cells isolated from blood in tilapia in control group and groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

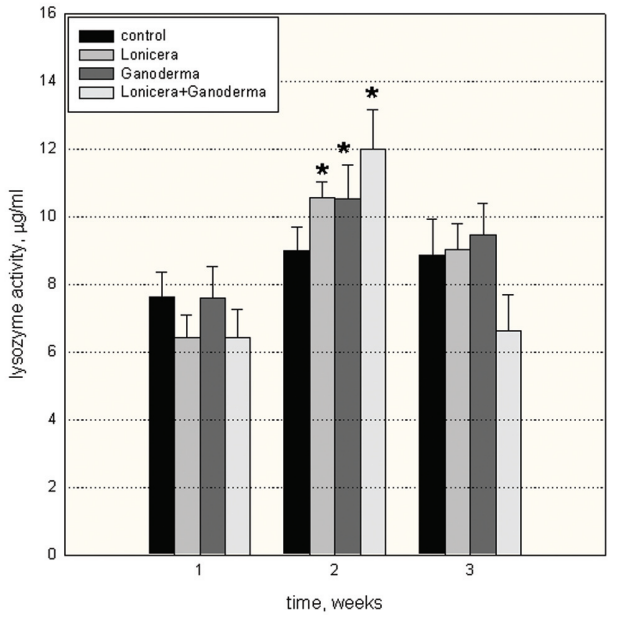

Figure 3. Changes in plasma lyzosyme activities in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

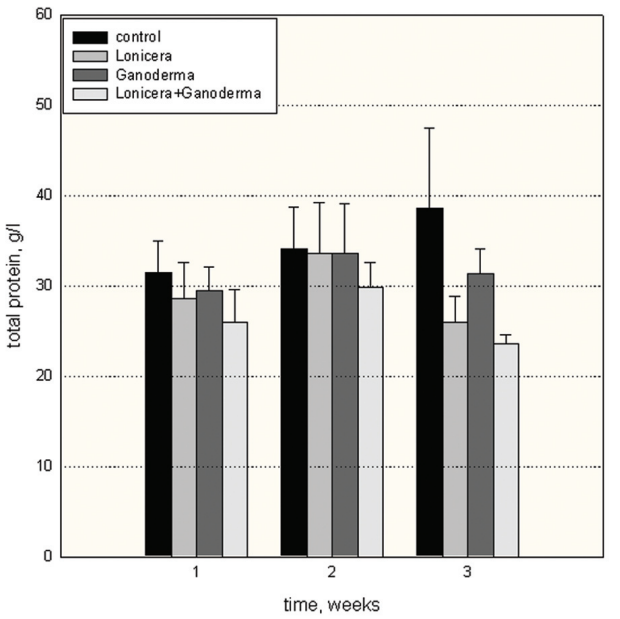

Figure 4. Changes in plasma plasma total protein values in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

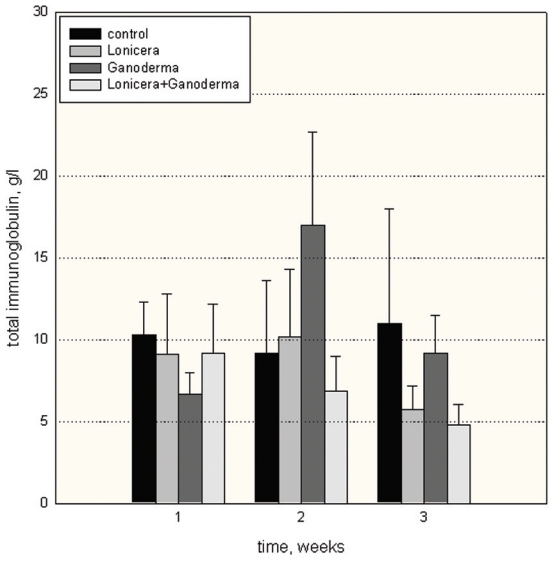

Figure 5. Changes in plasma plasma total immunoglobulin values in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

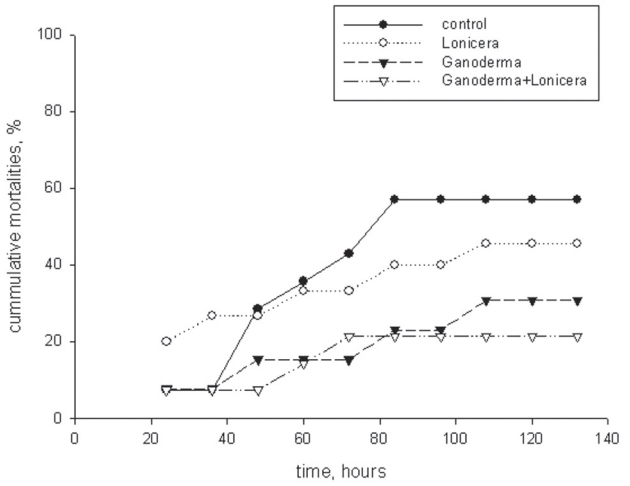

Figure 6. Cumulative mortalities (%) of tilapia in control and in groups fed diets containing different kind of herbs and over a 6 days after artificial challenging with *Aeromonas hydrophila.* Thirty fish from each group were used.

DISCUSSION

In this study, extracts of two Chinese herbs from Ganoderma **(***Ganoderma lucidium*) and from Lonicera (*Lonicera japonica*) were chosen because of their recorded ability to enhance the activity of the immune system. *Ganoderma lucidium* is a traditional Chinese medicine used for the prevention and treatment of various human diseases in China and other Asian countries (Lin, 2001). It has been shown that an aqueous extract from *G. lucidum* will promote phagocytosis by macrophages in mice immunosuppressed by cyclophosphamide (Tang, 2000; Wang *et al.*, 2003), it will stimulate proliferation of lymphocytes induced by concanavalin A or lipopolysaccharide and influence gene expression of cytokines (Wang *et al.,* 1997). The second herb, *L. japonica* has been known as an anti-inflammatory agent and used widely for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis (Lee *et al.,* 1998). It has been reported that Lonicera significantly increased blood neutrophil activity and promoted phagocytosis by the neutrophils in bovine at the correct concentration (Hu *et. al*., 1992).

The results from this study showed that both *Ganoderma* and *Lonicera* were able to modulate some parameters of the innate immune system of tilapia. The non-specific defence mechanisms of fishes include neutrophil activation, production of peroxidase and oxidative radicals, together with initiation of other inflammatory factors (Ellis, 1977; Ainsworth *et al.,* 1991). In this study fish fed with herb extracts showed an elevated phagocytosis over the whole period of the experiment. Fishes treated with immunostimulants usually show enhanced phagocytosis. Several studies have reported that oral administration of yeast products (MacroGard; Vitastim; *Saccharomyces cerevisiae*), (Aisworth *et al.,* 1994; Siwicki *et al.,* 1994; Jeney *et al.,* 1997), chitin (Sakai *et al.,* 1992), plant extracts, such as ginger increased the phagocytic capability of the cells in rainbow trout (Dügenci *et al.,* 2003) and extracts of four Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica, Lonicera japonica*) increased phagocytosis of white blood cells of crucian carp (Chen *et al.*, 2003).

Total lysozyme is a measurable humoral component of the non-specific defence mechanism, and while reports on modulation of the lysozyme activity in fishes are rare, increased values have been recorded by various authors after activation of the immune system with immunomodulants (Engstadt and Robertsen*.,* 1993; Siwicki *et al.,* 1994; Thompson *et al.,* 1995) and by feeding four different Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica Lonicera japonica*) (Chen *et al.,* 2003). In our previous study it was shown, that Astragalus enhanced lysozyme activities in tilapia during the whole period of the experiment when fed low (0.1%) and medium (0.5%) doses of herbs, while no effect was found when fish were fed with Scutellaria (Yin *et al.,* 2006).

In this study we used the reduction of ferricytochrome *c* to determine extracellular superoxide anion. Jeney *et al.,* (1997) showed that extracellular activity was very high in fish fed with dietary glucan. Rainbow trout fed with ginger (*Zingiber officinale*) extract had significantly higher extracellular activity of phagocytic cells in blood (Dügenci *et al.,* 2003). However, in this study we could not detect differences in respiratory burst activity in fish nor in our previous studies, when the tilapia were fed with Astragalus extract. Respiratory burst activity of phagocytic cells was not elevated and in the case of

fish fed with Scutellaria there was significant inhibition of extracellular superoxide anion production (Yin *et al.,* 2006). It was shown that in trout fed with nettle and mistletoe extracts the production of extracellular superoxide anion was of a similar level to that in the control fish (Dügenci *et al.,* 2003).

Total protein level and total immunoglobulin was not affected by feeding tilapia with herbs. Immunostimulants usually do not affect total protein levels, but fish with nutritional deficiencies may show lower plasma total protein levels (Siwicki *et al.,* 1994). On the other hand a decrease of total protein level in Russian sturgeon plasma was found after treatment of fish with glucan, chitosan and finnstim by immersion (Kolman *et al.,* 1998). Immunostimulants may raise the total immunoglobulin levels. Sturgeon treated with epin by immersion showed a higher concentration of total immunoglobulin (Kolman, 2001), whereas when chitosan, glucan and finnstim were administered by immersion the level of total immunoglobulin was decreased (Kolman *et al.,* 1998).

After challenge with *A. hydrophila* survival of fish fed with herb extracts was improved when compared with the control group. The survival was further enhanced in the group fed the Ganoderma extract supplement and when both herbs were used together. It is possible that this is the result of enhancement of some components of non-specific immune system of the fish by Ganoderma and a combination of Ganoderma and Lonicera. There is strong evidence that feeding glucans can modify the activity of the innate immune system of fish and increase the disease resistance in several fish species (Anderson, 1992; Sakai, 1999; Raa, 2000). Feeding carp with chitosan (1%) and levamisole (250 mg/kg of diet) also increased survival of fish following challenge with *A. hydrophila* (Gopalakannan and Arul, 2006).

The results of this study showed that feeding tilapia with *Ganoderma* and *Lonicera* alone or in combination enhanced phagocytosis by blood phagocytic cells during the whole experimental period and stimulated lysozyme activity after two weeks, but not respiratory burst activity of phagocytic blood cells, total protein or total immunoglobulin in plasma. Both herbs when used alone or in combination increased the survival of fish after challenge with *A. hydrophila.* Thus, it can be concluded that the herb extracts added to diets act as immunostimulants and appear to improve the immune status and disease resistance of fish.

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REFERENCES

Ainsworth, A.J., Dexiang, C. and Waterstrat, P.R. 1991. Changes in peripheral blood leukocyte percentages and function of neutrophils in stressed channel catfish. *J. Aquatic Anim. Health* 3:41-47.

Ainsworth, A.J., Mao, C.P. and Boyle, C.R. 1994. Immune responses enhancement in channel catfish, *Ictalurus punctatus,* using β-glucan from *Schizophyllum commune,* pp. 67-81. *In* Stolen, J.S. and Fletcher, T.C. (eds.). Modulators of Fish Immune Responses. Vol. I, SOS Publications, Fair Haven, NJ.

Anderson, D.P., 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. *Ann. Rev. Fish Dis*. 2:281-307.

Auro de Ocampo A. and Jimenez, E.M. 1993. Herbal medicines in the treatment of fish diseases in Mexico. *Veterinaria Mex*. 24:291-295.

Chansue, N., Ponpornpisit, A., Endo, M., Sakai, M and Satoshi, Y. 2000. Improved immunity of tilapia *Oreochromis niloticus* by C-UP III, a herb medicine. *Fish Pathol.* 35:89-90.

Chen, X., Wu, Z., Yin, J. and Li, L. 2003. Effects of four species of herbs on immune function of *Carassius auratus gibelio*. *J. Fish. Sci. of China* 10:36-40 (in Chinese).

Dey, R. K. and Chandra, S. 1995. Preliminary studies to raise disease resistant seed (fry) of Indian major carp *Catla catla* (Ham.) through herbal treatment of spawn. *Fish Chimes* 14: 23-25.

Direkbusarakom, S., Herunsalee, A., Yoshimizu, M. and Ezura, Y. 1996. Antiviral activity of several Thai traditional herb extracts against fish pathogenic viruses. *Fish Pathol*. 31:209-213.

Dügenci, S.K., Arda, N. and Candan, A. 2003. Some medicinal plants as immunostimulant for fish. *J. Ethnopharmacol*. 88:99-106.

Ellis, A.E. 1977. The leukocytes of fish: A review. *J. Fish Biol*. 11:453-491.

Engstadt, R. and Robertson, B. 1993. Recognition of yeast wall glucan by Atlantic salmon (*Salmo salar*). *Dev. Comp. Immunol.* 17:319-330.

Gopalakannan, A. and Arul, V. 2006. Immunododulatory effects of dietary intake of chitin, chitosan and levamisole ont he immune system of *Cyprinus carpio* and control of *Aermonas hydrophila* infection in ponds. *Aquaculture 225:179-187.*

Hu, S, Cai, W., Ye, J., Qian, Z. and Sun, Z. 1992. Influence of medicinal herbs on phagocytosis by bovine neutrophils. Zentralbl Veterinarmed Reihe A 39:593–599.

Jeney, G., Galeotti, M., Volpatt, D., Jeney, Z. and Anderson, D.P. 1997. Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. *Aquaculture* 154:1-15.

Kolman H., Siwicki, A.K. and Kolman, R. 1998. The effect of natural immunomodulators applied in immersion on non-specific immune responses in Russian sturgeon (*Acipenser güldenstadti* Brandt). *Arch. Polish Fish.* 6:391-410.

Kolman, H. 2001. The humoral effects of epin in siberian sturgeon (*Acipenser baeri* Brandt). *Arch. Polish Fish*. 9:61-69.

Lee, S.J., Son, K.H., Chang, H.W., Kang, S.S., Kim, H.P. 1998. Anti-inflammatory activity of *Lonicera japonica*. *Phytoth. Res*. 12:445-447.

Lin, Z.B. 2001. Modern Research of Ganoderma, pp. 284-309. *In Lin, Z.B. (ed.). Beijing Med. Univ. Press, Beijng.*

Logambal, S.M. and Michael, R.D. 2000. Immunostimulatory effect of azadirachtin in *Oreochromis mossambicus* (Peters). *Indian J. of Exp. Biol*. 38:1092-1096.

Luo, R. 1997. Induction of immunity substance in *Penaeus Chinensis* by Chinese herbal medicine. *Oceanlogia et Limnologia Sinica* 28:573-577 (in Chinese).

Miyazaki., T. and Jo, Y. 1985. A histopathological study of motile aeromonad diseas in ayu, *Plecoglosis altivelis*. *Fish Pathol. 20:55-60.*

Plumb, J.A. 1999. Health maintenance and principal microbial diseases of cultured fishes. Iowa State University Press, Ames, Iowa. 328 p.

Raa, J. 2000. The use of immune-stimulants in fish and shellfish feeds, pp. 47-56. *In* Cruz-Suarez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Olvera-Novoa, M.A. y Civera-Cerecedo, R. (eds.). Advance en Nutricion Acuicola V. Memorias del V Simposium Internacional de Nutrcion Acouicola. 19-22 Noviembre, 2000. Merida, Yucatan, Mexico.

Rahman, M.H., Kusuda, R. and Kawai, K. 1997. Virulence of starved *Aeromonas hydrophila* in cyprinid fish. *Fish Pathol. 32:163-168.*

Sakai, M., Kamiya, H., Ishii, S., Atsuta, S. and Koybayashi, M. 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhunchus mykiss*, pp. 413-417. *In* Shariff, M., Subasinghe, R.P. and Arthur, J.R. (eds.). Diseases in Asian Aquaculture I. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Sakai, M. 1999. Current research status of fish immunostimulants. *Aquaculture* 172:63-92.

Sankaran, K. and Gurnani, S. 1972. On the variation in the catalytic activity of lysozyme in fishes. *Ind. J. Biochem. Biophys.* 9:62-165.

Secombes, C.J. 1990. Isolation of salmonid macrophages and analysis of their killing activity, pp. 137-154. *In* . Stolen, J.S., Anderson, D.P., Robertson, B.S. and van Muiswinkel, W.B. (eds.) Techniques in Fish Immunology. SOS Publications, Fair Haven.

Seeley, K.R., Gillespie, P.D. and Weeks, B.A. 1990. A simple technique for the rapid spectrophotometric determination of phagocytosis by fish macrophages. *Marine Env. Res*. 30:123-128.

Siwicki, A.K., Anderson, D.P. and Rumsey, G.L. 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Vet. Immunol. Immunopathol*. 41:125-139.

Tang X. 2000. Effect of aqueous extract of *Ganoderma leucidum* on the immune function of mice. *TCM Res. 13:8-10.*

Thompson, K.D., Cachos, A. and Inglis, V. 1995. Immunostimulating effects of glucans and oxytetracycline in rainbow trout, *Oncorhynchus mykiss,* on serum lysozyme and protection, pp. 433-439. *In* Shariff, M., Subasinghe, R.P. and Arthur, J.R. (eds.). Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Wang K., Wu, G. and Dai, S. 2003. Study on the immunological effect of the aqueous extract from Guangxi *Ganoderma leucidum in the mice. J. Guangxi Med. Univ. pp. 871-874.*

Wang, S.Y., Hsu, M.L., Hsu, H.C., Tseng, C.H., Lee, S.S., Shiao, M.S. and Ho, C.K. 1997. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int. J. Cancer* 70:699-705.

Yin, G.., Jeney, G., Racz, T., Xu, P., Jun, X. and Jeney, Z. 2006. Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non-specific immune response of tilapia, *Oreochromis niloticus*. *Aquaculture* 253:39-47.